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# TOXICOKINETICS AND TOXICITY OF SEDIMENT-ASSOCIATED PYRENE TO *LUMBRICULUS VARIEGATUS* (OLIGOCHAETA)

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**Abstract** – The oligochaete *Lumbriculus variegatus* has been proposed for whole-sediment bioassays to assess sediment contamination. Our work examines *Lumbriculus variegatus* exposure to pyrene-dosed Lake Michigan sediment at 0.4 ng $g^{-1}$ and 64, 132, 206, and 269 $\mu g\ g^{-1}$. Both bioaccumulation and survival were followed to enhance *Lumbriculus variegatus* development as a bioassay organism. *Lumbriculus variegatus* accumulated sediment-associated pyrene rapidly and achieved apparent steady state within 48 to 168 h. The pyrene uptake clearances ($k_s$, g sed $g^{-1}$ animal $h^{-1}$) ranged from 0.039 to 0.132 and decreased with increasing pyrene concentration. At high pyrene concentrations, the worms avoided the sediment, which reduced accumulation and likely minimized the mortality response. In addition, the effect of organism loading on bioaccumulation was determined at different animal densities, 1:10, 1:50, and 1:100 g dry weight *Lumbriculus variegatus* : g sediment organic carbon, and a sediment pyrene concentration of 0.4 ng $g^{-1}$. Surprisingly, the bioaccumulation declined as organism density decreased. Pyrene elimination was rapid in clean sediment ($k_e = 0.026 \pm 0.002\ h^{-1}$) but was much slower in water ($k_e = 0.0043 \pm 0.0007\ h^{-1}$). Bioavailability apparently declined for exposures in sediment stored 1.5 months, based on the estimate of $k_e$ from nonlinear regression compared to direct measures of elimination. The apparent decline was attributed to both a decline in lipid content during the experiment and changes in pyrene bioavailability. Finally, for bioaccumulation studies, gut purging at a set time (e.g., 24 h) may result in an underestimate of contaminant concentration in organisms. An elimination study with extrapolation to the initial body burden can ensure that biases due to incomplete elimination of gut contents and body burden losses during the purging process are minimized.

**Keywords** – Toxicokinetics *Lumbriculus variegatus* Sediments Particle size Pyrene

## INTRODUCTION

It is well documented that sediments are a sink for hydrophobic organic pollutants discharged into the aquatic environment. Although bound mostly by sediment particles, these pollutants can be bioavailable to benthic organisms. Thus, sediment-associated contaminants can be toxic to benthic organisms or introduced in significant levels into the aquatic food chain. The effects of sediment-bound contaminants have been studied both by field survey techniques to detect changes in community structure [1–4] and by different lab bioassays [5,6].

Burton et al. [5] listed eight criteria to be considered when selecting test organisms for sediment toxicity assays, including their behavior in sediment (e.g., feeding); sensitivity to test materials; ecological relevance; geographical distribution; taxonomic relation to indigenous animals; acceptability for use in toxicity assessment; availability; and tolerance to natural geochemical sediment characteristics such as grain size. To these, additional criteria for bioaccumulation and subchronic assays can be included: the utility for assessing chronic end points; the incorporation of all relevant routes of exposure; and sufficient size for assessing bioaccumulation [7]. Although various bioassays have been developed to assess the toxicity and bioaccumulation potential of contaminants in sediments [8], most of them do not meet all of the above criteria.

Oligochaete worms like *Lumbriculus variegatus* meet most of the above test criteria and are easy to culture and handle in lab conditions [7]. Oligochaetes have been employed both in field surveys as bioindicators of pollution or trophic status [1,9,10] and in lab toxicity tests. To evaluate the toxicity of both single compounds and mixtures [11,12], oligochaete species are appropriate for measuring sublethal end points such as growth and reproduction [13,14] and behavioral responses such as sediment avoidance or feeding rate [15–17].

Among the bioassays proposed with *L. variegatus* [18] is their use as a standard freshwater bioaccumulation organism [19]. In such bioaccumulation procedures for sediment-ingesting benthic animals, a 12- or 24-h purging period in clean water [20,21] or in clean sediment [22,23] is often conducted before tissue analysis. This procedure is performed because sediment in the animals' gut is thought to contribute significantly to the analyzed body burden, especially in the case of metals [24,25]. However, within 24 h some animals can eliminate a considerable portion of their body burden. Further, some data show that in the case of chlorinated hydrocarbons, gut contents may not contribute significantly to measured body burdens [26].

Due to the interest in using oligochaetes for both bioaccumulation and toxicity bioassays, and the particular interest in using *L. variegatus* to evaluate the bioaccumulation of

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organic compounds in freshwater sediments, we measured the toxicity of pyrene to *L. variegatus* in Lake Michigan sediment. Further, we measured effects of pyrene on the growth and behavior of these animals and compared the uptake kinetics of radiolabeled pyrene at different sediment concentrations and different animal densities. The elimination of pyrene was measured in water and sediment to assess the role of gut contents and elimination media in this species.

## MATERIALS AND METHODS

### *Animals and environmental samples*

*Lumbriculus variegatus* as reared in 37-L glass aquaria containing well water under low-intensity (20 lx), gold fluorescent light ($\lambda > 500$ nm) with photoperiod of approximately 10:14 h light:dark at 23 ± 2°C. Shredded and presoaked unbleached paper towels were used as a substrate in the aquaria. A flow of 8 to 10 L fresh water per day was passed through the aquaria, and animals were fed with Trout Chow (Purina Brand®, St. Louis, MO; ≈3 g) three times per week. The water used for culture was well water that has a nominal pH of 7.2, hardness of 470 mg $L^{-1}$ as $CaCl_2$, and alkalinity of <1 mg $L^{-1}$ as $CaCO_3$ (D. O'Connor, personal communication).

Lake Michigan sediment was obtained by Ponar grab at 45 m depth about 8 km southwest of Grand Haven, Michigan (43°02.2′N, 86°21.9′W). The sediment was sieved at 1 mm to remove animals and large debris, and kept in the dark at 4°C. This sediment has a low background concentration of PAHs (pyrene about 250 ng/g dry weight) [27]. The sediment was stored for less than one month before use. Water used throughout the work was Lake Michigan surface water stored in the dark at 4°C before use. The water has a pH of 8, hardness of 139.3 mg $L^{-1}$ as $CaCO_3$, and alkalinity of 2.15 meq $L^{-1}$ [28].

### *Chemicals*

Pyrene ([4,5,9,10-$^3$H], specific activity 25.2 Ci/mmol, lot no. CLS-91-345-25-12, Chemsyn Science Laboratories, Lenexa, KS) was dissolved in acetone, and radiopurity was determined to be 99.3% using TLC and liquid scintillation counting (LSC). The TLC was performed on silica-gel plates with the hexane:benzene 80:20 (v/v) solvent system. The nonradiolabeled pyrene (Aldrich Chemical, Milwaukee, WI) was used as purchased. All preparative and analytical procedures as well as bioassays were performed under gold fluorescent lights ($\lambda > 500$ nm) to avoid pyrene photodegradation.

### *Sediment dosing*

For the toxicity experiment, the sediment was suspended in an aqueous slurry of 2,000 g wet sediment (dry-to-wet-weight ratio 0.6) in 2 L lake water. The pyrene was added in 4 ml acetone for all treatments, and the control sediment (untreated Lake Michigan sediment) was treated with the same amount of acetone. The concentration of nonradiolabeled pyrene stock solution (60 mg/ml) was made by dissolving 1.5 g pyrene in 25 ml acetone in a volumetric flask. The appropriate volume (up to 4 ml) containing the needed compound was placed in a small vial, and 10.8 μCi of [$^3$H]pyrene was added to each vial. The stocks were mixed, and three 2-μl samples were taken for LSC. The remainder was added dropwise to the sediment slurry. Four different treatment concentrations were prepared: 64, 132, 206, and 269 μg $g^{-1}$, based on the measured radioactivity and the new specific activity for each dose. For the treatments requiring <4 ml of stock solution, sufficient additional acetone was added to the vial to make the amount of carrier constant. The water–sediment mixtures were stirred at room temperature for 4 h and held at 4°C overnight. Four hours of mixing was sufficient for initial sorption, based on the C.V. for the sediment pyrene concentration of <7%. The overlying water was assayed for total radioactivity for mass balance and decanted from the sediment. The sediments were mixed with 300 ml fresh lake water and again allowed to stand overnight. This procedure was repeated three times to ensure the removal of water-soluble carrier. For this sediment, overnight settling produced a clear overlying water and was considered adequate settling for the washing procedure.

For the toxicokinetic experiments, trace pyrene concentration sediments (0.4 ng $g^{-1}$) were prepared by dosing 6,000 g wet sediment with [$^3$H]pyrene only. A solution of 80 μCi of pyrene in 100 μl acetone was added dropwise to sediment slurry (3,000 g wet sediment and 1,500 ml lake water) in a 4-L beaker, while the mixture was stirred vigorously for 4 h at room temperature and kept at 4°C for 3 d. The overlying water was assayed for total radioactivity for mass balance and decanted. The sediments from two beakers were combined and thoroughly mixed. Any remaining amount of water-soluble carrier should have had only a minimal effect on the pyrene partitioning to the sediment [29].

After 4 d each pyrene treatment was sampled for contaminant concentration and wet- and dry-weight measurements. The sediments were held under lake water for one-and-a-half months in the dark at 4°C. The one-and-a-half-month equilibration was thought to be sufficient to permit the bioavailable fraction of the sediments to stabilize [30].

### *Toxicity assay*

After the storage period, the overlying water was decanted, the sediment was mixed to visual homogeneity, and 40 g wet sediment was distributed to each 50-ml glass exposure beaker. Lake water was then carefully added with minimal sediment disturbance to each beaker. On the following day, 10 test organisms were carefully pipetted into each beaker, yielding a ratio of 1:10 animal dry weight:sediment organic carbon (SOC). These groups of 10 *L. variegatus* were exposed at 23 ± 1°C to each pyrene concentration (64, 132, 206, and 269 μg $g^{-1}$). Triplicate samples were removed at 7, 24, 48, 96, and 168 h. Thus, the total number of beakers was 15 per treatment concentration, including an untreated control sediment. The oxygen concentration was not monitored, but every other day the overlying water was carefully pipetted out of the beakers and new water added, avoiding sediment disturbance. Previous work with this sediment under static conditions suggested that oxygen would not be limited with water renewal [31,32]. This suggestion was confirmed with *L. variegatus*, for which the overlying water was changed at a less frequent interval and remained at >60% of saturation (see "Bioaccumulation assay").

Three beakers from each treatment concentration were sampled randomly at each time point. At these time points

1. The number of *L. variegatus* not burrowed into the sediment was recorded to check for possible sediment avoidance behavior.
2. The number of surviving *L. variegatus* was recorded for mortality determination.
3. The surviving animals were divided into two groups, weighed, and placed in scintillation cocktail to measure the radioisotope concentration for toxicokinetic calculations.
4. The sediment in each beaker was sampled for dry-to-wet-weight ratios and radioisotope concentration (in triplicate).
5. At the last sampling time, six individuals per treatment concentration were taken for microgravimetric lipid analyses [33].

*Bioaccumulation assay*

For the accumulation experiment at a trace concentration of 0.4 ng $g^{-1}$ pyrene, two sets of beakers were prepared: one similar to the toxicity experiment conducted in 50-ml beakers (40 g wet sediment and 10 animals), having a ratio of 1:10 animal dry weight:SOC (10,400 individuals/$m^2$); and another conducted in 200 g wet sediment in 400-ml beakers, including 10 animals, yielding a ratio of 1:50 animal dry weight:SOC (2,600 individuals/$m^2$). These groups of 10 *L. variegatus* were exposed at 23 ± 1°C for each animal-dry-weight-to-SOC ratio. Samples were again taken in triplicate at 15, 48, 96, 168, and 336 h for both ratios and at 22, 28, and 58 d for the 1:50 ratio only. Thus, the total number of beakers was 15 for the 1:10 ratio and 24 for the 1:50 ratio. Unspiked controls were sampled at 336 h for both exposure ratios and at 28 and 58 d for the 1:50 ratio. The oxygen concentration was monitored in this experiment, and it remained over 60% of saturation for the duration of the assay. The overlying water was changed once per week in the beakers. Besides these two sets of exposures, six 50-ml beakers with 40 g wet sediment and only one animal in each beaker were sampled at 336 h to determine bioaccumulation at animal density of a ratio of 1:100 animal dry weight:SOC (1,040 individuals/$m^2$).

Three beakers from both exposure ratios were randomly sampled at each time point. The number of surviving animals in each beaker was recorded, and animals were divided into two groups, weighed, and placed in scintillation cocktail to measure the radioisotope concentration for toxicokinetic calculations. Also, the sediment in each beaker was sampled for wet- and dry-weight ratio and radioisotope concentration (in duplicate). At 28- and 58-d sampling times, subsamples of sediment were analyzed for pyrene radiopurity (see "Analyses").

*Elimination*

For the pyrene elimination experiment, 150 *L. variegatus* were exposed for 7 d in 300 g wet weight of [$^3$H]pyrene-treated sediment (1:5 animal dry weight:SOC, 38,900 individuals/$m^2$) from the same treatment concentration used in the bioaccumulation assay. After exposure, animals were gently sieved from the sediment and divided randomly into 11 groups. One group was taken for radioisotopic analysis (divided into five subsamples), five groups were placed in 40 ml lake water, and five groups were placed in uncontaminated Lake Michigan sediment for elimination studies. Samples were taken at 12, 24, 48, 72, and 96 h, one group at a time from both elimination media. Animals were weighed and radioisotope concentrations were determined.

*Analyses*

At the end of each time interval, animals were gently sieved from the sediment, rinsed in filtered lake water, blotted dry, weighed (Cahn [Cerrites, CA] 4700 electrobalance), and placed in scintillation cocktail (Research Products International [Prospect, IL], 3a70B). Tritium activity was counted after 2 d with an LKB (Bromma, Sweden) 1217 liquid scintillation counter. The data were corrected for quench using the external standards ratio method after correcting for background. Treated wet sediment samples were taken in triplicate for contaminant concentration, dry-to-wet-weight ratios, and SOC. The dry-to-wet-weight ratios were determined by weighing a wet sediment sample and drying at 90°C to constant weight. Contaminant concentration in sediment was determined by placing approximately 100 mg wet sediment into 12 ml scintillation cocktail, sonicating (Tekmar [Cincinnati, OH] high-intensity sonic disrupter) for 2 min to maximize the extraction of pyrene, and measuring [$^3$H]activity 2 d after sonication.

The SOC content was determined by drying sediment samples, treating 100 mg dry sediment with 1 N HCl to remove carbonates, redrying, and analyzing organic carbon on a Perkin Elmer (Norwalk, CT) 2400 CHN elemental analyzer.

The sediment samples (5–10 g wet weight) for the pyrene purity check were extracted twice with ethyl acetate–acetone mixture (4:1, v/v) and twice with benzene. The extracts were combined, dried over $Na_2SO_4$, and rotary evaporated to a few milliliters. The rest of the solvent was transferred into a test tube and further evaporated to about 100 $\mu$l under a gentle stream of nitrogen for TLC analysis. Remaining solvent was introduced onto silica TLC plate (E. Merck [Darmstadt, Germany], 250-$\mu$m coating), some nonradiolabeled pyrene was added over the sample, and the plate was developed with hexane:benzene (4:1, v/v) solvent. After the run, the pure pyrene spot was marked under UV light, and the plate was analyzed for radioactivity by scraping 2-cm segments of the silica gel and counting them in 12 ml scintillation cocktail.

Sediment particle-size distribution was determined by a modified sedimentation technique [34,35]. Approximately 40 g wet sediment was first wet sieved using filtered (0.3 $\mu$m; Gelman Sciences [Ann Arbor, MI], glass fiber, type A/E) Lake Michigan water through 420-, 105-, and 63-$\mu$m standard sieves. Materials remaining on the sieves were collected in beakers. Triplicate samples were taken for LSC, and the remainder was dried to constant weight at 90°C for mass and SOC analyses. Material passing through the 63-$\mu$m sieve was mixed with 1.0 L filtered Lake Michigan water in a graduated cylinder at room temperature. Samples (25 ml) from the sediment suspension were taken at 20 cm depth at 0, 120, 240, and 600 s after mixing. After 1,200 and 4,600 s, water samples were taken at a depth of 10 cm. The sampling times and

depths were calculated by Stoke's law using 2.6 as the specific gravity of the particles [34]. This calculation yields the settling rates for particles of different sizes, assuming a uniform density and spherical dimensions. From each sample, three 2-ml aliquots were analyzed via LSC. The rest of the sample (19 ml) was dried to constant weight at 90°C for mass and SOC determinations.

*Calculations*

Accumulation data were fit to a two-compartment model [36]:

$$C_a = \frac{k_s \cdot C_s}{k_e}(1 - e^{-k_e t}), \quad (1)$$

where the amount of pyrene at each concentration is represented by the amount of tritium activity expressed as disintegrations per minute (dpm), based on the specific activity of the dosing solution after isotopic dilution. Thus,

$C_a$ = the pyrene concentration in the animals (dpm $g^{-1}$ wet weight)
$C_s$ = the pyrene concentration sediment (dpm $g^{-1}$ dry weight)
$k_s$ = the uptake clearance of pyrene from sediment (g dry sediment $g^{-1}$ wet organism $h^{-1}$)
$k_e$ = the elimination rate constant of pyrene ($h^{-1}$) in sediment
$t$ = time (h).

To be valid, it was assumed that the bioavailable concentration of pyrene in the sediment remained constant and biotransformation of the pyrene by *L. variegatus* was sufficiently slow and would not result in significant loss over the time course of the experiment. Subsequent studies have confirmed the inability of *L. variegatus* to biotransform selected PAHs at a rate sufficient to be measured [37].

The data from the elimination experiment were fit to a first-order decay:

$$C_a^{(t=t)} = C_a^{(t=0)} \cdot e^{-k_e t}, \quad (2)$$

where $C_a^{(t=0)}$ = pyrene concentration in the animals at the beginning of the elimination experiment (dpm $g^{-1}$ wet weight).

*Statistics*

Student's $t$ test was used when comparing means or slopes of regression lines. Differences between means and slopes were considered significant when $p < 0.05$. General linear method (GLM) for least-squares regression and nonlinear curve fitting (NLIN) for accumulation curves were used in SAS® [38]. Errors are standard deviations for means and standard errors for slopes and intercepts of regression lines.

## RESULTS

*Sediment*

Pyrene sediment concentrations exhibited no significant change during the toxicity exposure, and the average concentrations were 64 ± 3 ($n$ = 15), 132 ± 6 ($n$ = 15), 206 ± 9 ($n$ = 15), and 269 ± 14 $\mu g\ g^{-1}$ ($n$ = 15). The measured sediment dry-to-wet-weight ratio was 0.64 (±0.01, $n$ = 60). In the bioaccumulation assay, the pyrene sediment concentration remained steady during assay at 0.41 ± 0.01 ng $g^{-1}$ ($n$ = 54), and the concentrations did not differ statistically between exposures with different animal densities. Sediment samples extracted after 28- and 58-d exposures (1:50 ratio) had pyrene radioactive purity of 91.6% ($n$ = 2) and 92.7% ($n$ = 2), respectively, showing that no major breakdown of pyrene occurred during the long-term exposure. The sediment dry-to-wet-weight ratios were 0.68 (±0.01, $n$ = 30) and 0.69 (±0.01, $n$ = 48) in the ratios of 1:10 and 1:50 animal dry weight:SOC, respectively. The SOC content remained unchanged at every pyrene treatment concentration and averaged 0.44 ± 0.02% ($n$ = 76).

Particle sizes ranging from 420 to 43 $\mu$m made up 80% of the total dry weight of the sediment (Fig. 1). Size fractions smaller than 63 $\mu$m were verified via Coulter® (Toronto, Ontario) counter technique (unpublished data) to ensure the ac-

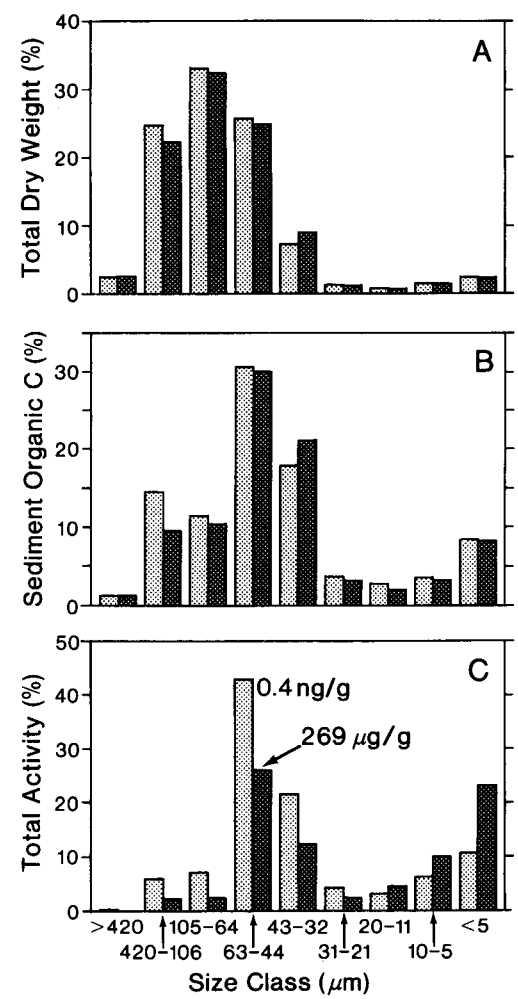


Fig. 1. Particle-size distribution of sediment mass (**A**), organic carbon (**B**), and pyrene content (**C**) in Lake Michigan sediment at 0.4-ng $g^{-1}$ and 269-$\mu g\ g^{-1}$ pyrene concentrations. Values represent the mean of two replicates.

curacy of the calculated particle sizes based on Stoke's law. The distribution of organic carbon present in sediment was slightly different from the particle-size distribution. Approximately 50% of SOC resided with the particles in the 63- to 31-$\mu$m range (Fig. 1). There was no difference in particle mass or SOC distributions between the 0.4 ng $g^{-1}$ and 269 $\mu$g $g^{-1}$ pyrene sediment concentrations. However, the pyrene distributions in these two sediments were different (Fig. 1). In the high-pyrene concentration (269 $\mu$g $g^{-1}$), small particles tended to sequester a greater portion of pyrene than in the trace concentration (0.4 ng $g^{-1}$). The relative OC-normalized concentrations of pyrene (OC-normalized pyrene concentration in a fraction divided by the OC-normalized pyrene concentration in whole sediment) in different fractions suggests the same (Fig. 2). In the trace concentration, the pyrene-relative OC-normalized concentrations were <1.0 in coarse fractions (>63 $\mu$m), suggesting that OC in these fractions does not bind pyrene as efficiently as the OC in smaller particles. In these smaller particles, the relative concentration is between 1.0 and 1.7, suggesting rather uniform pyrene distribution within the OC of the smaller (<63 $\mu$m) particles. At the high treatment concentration, particles <10 $\mu$m had more than three times higher OC-normalized pyrene concentrations than the OC-normalized concentration for the whole sediment (Fig. 2).

*Toxicity*

Pyrene was only slightly toxic to *L. variegatus* at the tested concentrations. After 7 d of exposure, mortality was 3, 7, and 17% at 132-, 206-, and 269-$\mu$g $g^{-1}$ treatment concentrations, respectively. One reason for the low acute mortality response may have been sediment avoidance. In high pyrene concentrations, animals did not burrow into the sediment as they did at lower concentrations and controls. After 7 d of exposure, 50 and 64% of the surviving animals were on the sediment surface of the 206- and 269-$\mu$g $g^{-1}$ treatment concentrations. At lower treatment concentrations and in the control sediment, all the animals were burrowed into the sediment. The EC50 value calculated by probit analyses for sediment avoidance after 7 d was 226 $\mu$g $g^{-1}$ (95% limits: 204–254).

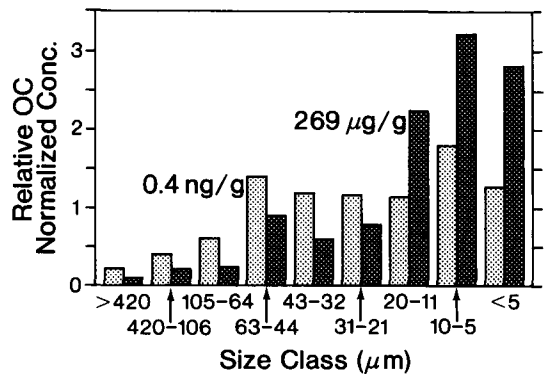


Fig. 2. Relative organic-carbon-normalized concentrations of pyrene (OC-normalized concentration in the particle-size fraction divided by the OC-normalized concentration in whole sediment) in Lake Michigan sediment at two concentrations.

The animals were not fed during the exposures, and the low OC content of the sediment apparently did not provide the quantity or quality of food equivalent to that of the culture conditions. The wet weight of the animals decreased in every treatment concentration, including the controls (Tables 1 and 2). However, animals in the high-pyrene treatment concentration lost more weight than the controls (Table 1)—the likely result of the avoidance behavior noted above—which suggests some growth effect of pyrene. Although the wet weight of animals and the total biomass per beaker decreased, the number of animals increased at trace concentration exposures after two weeks in the 1:50 and 1:100 ratio and 1:10 controls (Table 2), which suggests that the low nutrient environment was not of sufficient stress to preclude reproduction.

The high lipid content in the animals taken from the culture aquarium also indicated better nutritional conditions in the culture than in the Lake Michigan sediment (Table 3). In the toxicity experiment, lipid content decreased within a week in each pyrene treatment and in the control (Table 3). The control lipid content did not differ significantly from those of the treatments. With the long-term exposure, animal lipid

Table 1. Wet weight (mg; mean ± SD) of animals in different exposures during the first week

| Time (h) | Wet weight (mg) of worms exposed to various pyrene concentrations | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | 0.4 ng/g (1:10) | 0.4 ng/g (1:50) | 64 $\mu$g/g | 132 $\mu$g/g | 206 $\mu$g/g | 269 $\mu$g/g |
| 7 | 5.76 (0.60) | – | – | 5.44 (0.61) | 5.99 (0.15) | 5.75 (0.67) | 5.78 (0.67) |
| 17 | – | 6.37 (0.49) | 6.45 (0.38) | – | – | – | – |
| 24 | 5.94 (0.74) | – | – | 5.24 (0.57) | 5.33 (0.41) | 5.78 (0.95) | 5.27 (0.33) |
| 48 | 5.55 (0.95) | 6.45 (0.24) | 6.48 (0.70) | 4.85 (0.26) | 5.58 (0.79) | 5.04 (0.85) | 5.04 (0.47) |
| 96 | 4.92 (0.48) | 5.87 (0.67) | 5.92 (0.24) | 4.45 (0.41) | 4.74 (0.45) | 4.65 (0.67) | 4.07 (0.42) |
| 168 | 4.93 (0.23) | 5.26 (0.16) | 5.75 (0.47) | 4.69 (0.45) | 4.21 (0.46) | 4.58 (0.87) | 3.62 (0.29) |
| Slope[a] | −0.0065 | −0.0081 | −0.0040 | −0.0048 | −0.0103 | −0.0079 | −0.0133 |
| ±SE[a] | ±0.0019 | ±0.0015 | ±0.0018 | ±0.0023 | ±0.0019 | ±0.0024 | ±0.0019 |
| $r^2$[a] | 0.793 | 0.937 | 0.717 | 0.593 | 0.904 | 0.784 | 0.941 |
| $P$[a] | 0.043 | 0.032 | 0.153 | 0.128 | 0.013 | 0.046 | 0.006 |

At each time point, three beakers per treatment concentration were sampled, and animals in each beaker were weighed in two groups.
[a]For the linear regression of weight lost during the exposure.

Table 2. Mean number of animals in beakers and wet weight (±SD, $n = 3$) at different loading densities after exposure

| Ratio | Time (d) | No. of animals in beaker (SD) | $n$ | Wet wt.[a] (mg/worm) |
|---|---|---|---|---|
| 1:10 | 7 | 11.0 (1.0) | 3 | |
| | 14 | 10.3 (0.6) | 3 | 4.92 (0.36) |
| | 14 C[b] | 13.0 (1.3) | 6 | 4.25 (0.44) |
| 1:50 | 7 | 10.3 (0.6) | 3 | |
| | 14 | 14.0 (0.0) | 3 | 4.07 (0.57) |
| | 14 C | 14.7 (3.0) | 3 | 3.82 (0.10) |
| | 28 | 14.7 (0.6) | 3 | 3.35 (0.21) |
| | 28 C | 16.0 (2.6) | 3 | 3.66 (0.28) |
| | 58 | 15.0 (0.0) | 3 | 2.21 (0.05) |
| | 58 C | 15.3 (1.0) | 4 | 2.38 (0.05) |
| 1:100 | 14 | 1.2 (0.4) | 6 | 5.38 (1.08)[c] |
| | 14 C | 1.8 (0.4) | 6 | 4.15 (0.70)[c] |

At the beginning of exposure, the number of animals in a beaker was 10 at 1:10 and 1:50 animal dry weight:SOC, and one at 1:100 animal dry weight:SOC.
[a]Wet weight at $t = 0$ was 6.0 ± 0.55 mg/worm, $n = 5$.
[b]Control.
[c]$n = 6$.

Table 3. Animal lipid content at end of toxicity assay for different pyrene treatment concentrations and during bioaccumulation experiment (animals kept in untreated Lake Michigan sediment)

| Treatment/sample | Time (d) | Lipid % (SD) | $n$ |
|---|---|---|---|
| Toxicity assay | | | |
| Animals from | | | |
| Culture aquarium | 0 | 13.2 (4.3) | 8 |
| Control | 7 | 9.2 (0.9) | 3 |
| 64 μg/g | 7 | 8.4 (1.2) | 3 |
| 132 μg/g | 7 | 10.8 (1.6) | 3 |
| 206 μg/g | 7 | 9.0 (1.5) | 3 |
| 269 μg/g | 7 | 11.0 (1.4) | 3 |
| Bioaccumulation assay | | | |
| Animals from | | | |
| Culture aquarium | 0 | 11.1 (1.4) | 11 |
| 1:10 density | 14 | 4.9 (1.0) | 10 |
| 1:50 density | 14 | 5.1 (2.0) | 7 |
| 1:50 density | 28 | 4.9 (1.2) | 7 |
| 1:50 density | 58 | 6.3 (1.3) | 10 |
| 1:100 density | 14 | 6.4 (1.9) | 5 |

Sample density refers to the animal dry weight:SOC ratio. Each analyzed sample contained two to three dried worms (except 1:100 density, where worms were analyzed individually).

content decreased significantly from culture conditions after two weeks and remained lower during the rest of the experiment (Table 3). Animal density had a small effect on the lipid content of the animals (the higher the animal density, the lower the lipid content), but the differences were not statistically significant (Table 3).

*Bioaccumulation*

Pyrene was quickly accumulated by *L. variegatus* at each treatment concentration (Figs. 3 and 4). Apparent steady state was reached within 168 h, except at the 132-μg $g^{-1}$ treatment concentration, where the pyrene body burden continued to increase (Fig. 3). At the trace concentration (0.4 ng $g^{-1}$), the accumulation of pyrene reached a peak at 48 h and decreased after that, except for the 1,390-h sample, when body burden was the highest (Fig. 4A). The decline after 48 h was the same at both animal densities during the first two weeks.

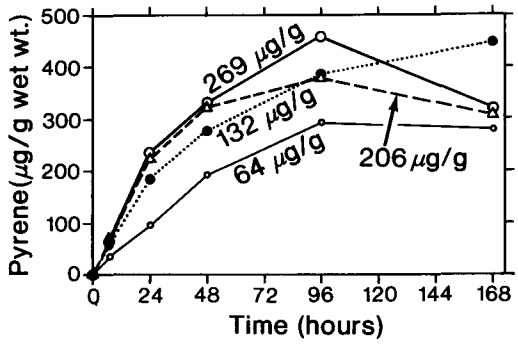


Fig. 3. Pyrene body burden in *Lumbriculus variegatus* during the acute toxicity assay.

The pyrene uptake clearances were calculated using either the organism wet weight or lipid-normalized body burdens, according to Equation 1. The $k_s$ values tended to decrease with increasing pyrene sediment concentrations (Table 4). This change in $k_s$ values across treatments demonstrates the

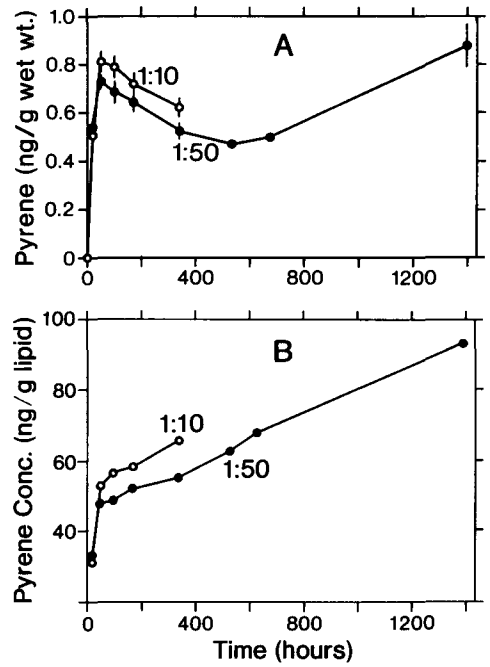


Fig. 4. Pyrene body burdens (**A**) and lipid-normalized body burdens (**B**) in *Lumbriculus variegatus* during the long-term exposure to 0.4-ng $g^{-1}$ sediment concentration.

Table 4. Uptake ($k_s$) and elimination ($k_e$) clearance constants (SE) for pyrene by *Lumbriculus variegatus* at different pyrene concentrations

| Concn. | $k_s$ | $k_e$ ($h^{-1}$) | $k_{sl}$ | $k_{el}$ ($h^{-1}$) |
|---|---|---|---|---|
| 0.4 ng/g[a] | 0.156 (0.029) | 0.101 (0.020) | 6.59 (0.46) | 0.053 (0.004) |
| 0.4 ng/g[b] | 0.130 (0.016) | 0.075 (0.010) | 5.93 (0.42) | 0.041 (0.003) |
| 64 μg/g[b] | 0.094 (0.009) | 0.019 (0.003) | 4.72 (0.42) | 0.013 (0.002) |
| 132 μg/g[b] | 0.066 (0.005) | 0.019 (0.002) | 3.11 (0.24) | 0.012 (0.002) |
| 206 μg/g[b] | 0.068 (0.008) | 0.040 (0.006) | 3.24 (0.33) | 0.030 (0.004) |
| 269 μg/g[b] | 0.051 (0.006) | 0.035 (0.005) | 2.50 (0.26) | 0.027 (0.004) |

Values are obtained by fitting Equation 1 to the organism wet-weight-normalized data ($k_s$, g dry sed. $g^{-1}$ wet organism $h^{-1}$) or to the lipid-normalized data ($k_{sl}$, g dry sed. $g^{-1}$ lipid $h^{-1}$).
[a]Animal dry weight:SOC = 1:50.
[b]Animal dry weight:SOC = 1:10.

conditional nature of these values. Animals in the ratio of 1:50 animal dry weight:SOC had slightly higher $k_s$ values than those in the 1:10 ratio (Table 4). However, after 7 and 14 d, the body burdens were inversely proportional to animal density (Fig. 5), and there was a statistical difference ($t$ test, $p < 0.05$) in body burdens between animal densities.

The decline in $k_s$ with increasing sediment concentration can potentially be attributed to both sediment avoidance and a difference in pyrene distribution among the particles. For the 0.4 ng $g^{-1}$ and 269 μg $g^{-1}$ pyrene in sediment, the $k_s$ values on a whole-sediment basis varied by a factor of 2.5. If the $k_s$ values are calculated on the pyrene concentration on the <20-μm particles, the relative difference between the two concentrations remains 2.1. However, if the $k_s$ values are calculated on the pyrene concentration in the 63- to 31-μm size class, then the $k_s$ values (0.201 and 0.131, respectively) vary by only a factor of 1.5. The reduction in range suggests that selective feeding is a major factor but that sediment avoidance remains important for the differences in exposure.

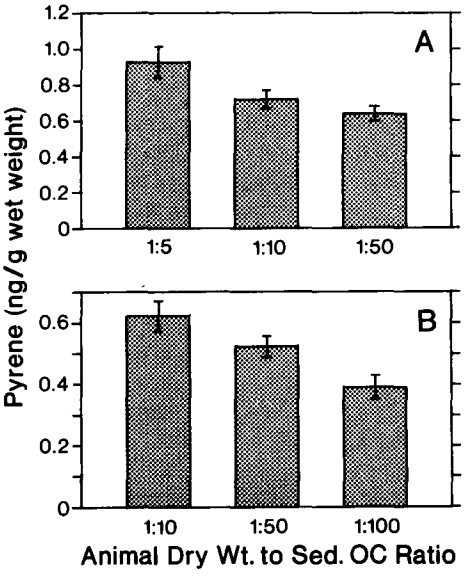


Fig. 5. Pyrene body burden in *Lumbriculus variegatus* after 7-d (**A**) and 14-d (**B**) exposures at 0.4-ng/g-dry-weight sediment concentration in different ratios of animal dry weight to sediment organic carbon.

*Elimination*

The elimination of pyrene was evaluated by measuring elimination in both sediment and water and by analyzing the nonlinear fit of the accumulation data (Eqn. 1). Measured pyrene elimination was rapid in the presence of sediment, and several half-lives occurred over the course of the 96-h experiment (Fig. 6). Under water-only elimination conditions, pyrene was eliminated much more slowly (Fig. 6). When the data were fit by linear regression to Equation 2, the elimination rate constants, $k_e$, were 0.0256 (±0.0016) $h^{-1}$ ($r^2$ = 0.93) and 0.0043 (±0.0007) $h^{-1}$ ($r^2$ = 0.62) for the sediment and water-only experiments, respectively. The corresponding half-lives ($t_{1/2}$) are 27.1 h in the presence of sediment and 161.2 h in the absence of sediment. When elimination for trace exposure (0.4 ng $g^{-1}$) was examined through nonlinear fit of Equation 1, the elimination rate constant was apparently much larger than the direct measurement (Table 4). The directly measured elimination was more comparable to the values of nonlinear regression at the high treatment concentrations (Table 4).

## DISCUSSION

Although oligochaete worms have been used in several sediment toxicity studies, this study is one of the few in which toxicokinetics were concurrently analyzed. This type of study is required to set suitable recommendations (exposure time, animal density, etc.) for toxicity assays with organisms and to provide better interpretation of toxicity bioassays and field contaminant concentrations in organisms.

The uptake clearances ($k_s$) of pyrene for *L. variegatus* are somewhat lower than those measured for another oligochaete worm, *Stylodrilus heringianus*, exposed to sediment-associated pyrene for sediment sampled from the same station as that used in this study [39]. The elimination rate constants for pyrene are similar in both species. The differences in $k_s$ values between the two species may be attributed to differences in the experimental conditions as well as species differences.

The observed sediment avoidance accounts primarily for the lower $k_s$ values at high pyrene concentrations. If animals

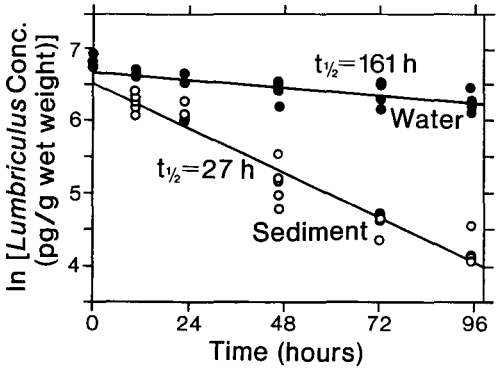


Fig. 6. Pyrene elimination by *Lumbriculus variegatus* in clean Lake Michigan sediment and in clean water. Regression equations for lines are sediment: $\ln(C_a) = 6.499\ (\pm 0.089) - 0.0256\ (\pm 0.0016) \times$ time, ($r^2 = 0.930$); and water: $\ln(C_a) = 6.659\ (\pm 0.041) - 0.0043\ (\pm 0.0007) \times$ time, ($r^2 = 0.621$).

avoid the sediment, they have reduced sediment particle ingestion and accumulation is reduced. Even if they are burrowed in the sediment, high contaminant concentrations may decrease their sediment ingestion rate [15,16] and thus reduce accumulation. In addition, the effect of pyrene concentration on the $k_s$ values (Table 4) can be related partly to the differential particle-size distribution of pyrene in the sediment (Figs. 1 and 2). This is confirmed by the reduction in the range of $k_s$ values when calculated on pyrene concentrations associated with the 31- to 63-$\mu$m particle-size fraction, compared to the range when total pyrene concentration was employed for the calculations. Compounds bound by the small size fractions (<20 $\mu$m) are not readily bioavailable to *Diporeia* spp., even when those particles are ingested [40]. In this study, pyrene was almost equally distributed in the SOC at the trace-concentration treatment, but at the highest pyrene treatment concentration, a much greater portion of pyrene was associated with the small particle sizes (Fig. 2). Thus, at the highest pyrene treatment concentration, more compound was in the nonbioavailable fraction. The relatively small reduction in the range of $k_s$ values when calculated using the pyrene concentrations for the <20-$\mu$m size fraction confirms the notion that pyrene associated with these particles is less available than that associated with the 31- to 63-$\mu$m size fraction. Currently, the uptake clearance calculation (Eqn. 1) uses the concentration in the whole sediment and may result in a lower $k_s$ value at the high pyrene concentration than in the low concentration, where distribution is equal through particle sizes. Both factors, sediment avoidance and differential distribution, are thought to contribute to the lower $k_s$ values obtained at high pyrene concentrations. Further, deciding which pyrene concentration of which fraction is appropriate to represent the bioavailable fraction for $k_s$ calculations remains unknown. However, the reduction in range observed when using the pyrene concentration on the 63- to 31-$\mu$m particles suggests that using whole-sediment concentrations is not representative of the bioavailable fraction.

The avoidance behavior and resultant reduction in flux into the worms limits bioaccumulation and subsequent mortality. The animal body burden actually peaked at 48 h and slightly decreased to 168 h at the two highest pyrene concentrations (Fig. 3). The ability of oligochaetes to detect contaminants and to avoid the sediment has been observed for *S. heringianus* and *Limnodrilus hoffmeisteri* with endrin-contaminated sediments at concentrations 50 to 150 times lower than 96-h LC50 concentrations [15]. This avoidance response was suggested as a potential subacute toxicity end point [15]. Similarly, some invertebrates seem able to detect some classes of compounds at toxic concentrations—for example, *Diporeia* spp. avoid PAH-contaminated sediment [31]—whereas other classes of compounds may not be detected—*Diporeia* spp. did not avoid toxic concentrations of chlorinated hydrocarbons [32]. As a result of the avoidance behavior, the relative bioaccumulation of pyrene was less for the high treatment concentrations than for the lower treatment concentrations and observed mortality was lower than expected. At the highest treatment concentration, the body burden peaked at 96 h, reaching 2.3 $\mu$mol $g^{-1}$, but did not remain that high. For nonpolar narcotics such as pyrene, this is the lower end of the observed concentration required to produce 50% mortality in aquatic organisms [36]. Thus, the 17% mortality at the highest treatment concentration was consistent with the observed internal concentration. Overall, the avoidance of contaminated sediment can modify accumulation and toxicity of sediment-associated contaminants. This behavior in oligochaetes and other organisms employed for bioaccumulation and toxicity testing must be reported to provide proper interpretation of test results. Failure to observe avoidance may lead the investigator to believe that a sediment is nontoxic (produces no observed mortality), when in fact the organism has not been exposed to the anticipated extent.

The inverse relationship between animal density and accumulated body burden was an unexpected phenomenon. At high animal densities (i.e., high ratios of animal dry weight to SOC), we expected that animals would deplete the available compound reserves and end up with lower body burdens. At higher animal densities, the porosity of the sediment may be increased, permitting greater pore-water volumes and greater uptake through this route. Alternatively, at the higher animal densities, the worms may deplete the best possible fraction of food in the sediment and then have to compensate by increasing the feeding rate to compete for the remaining food source. Increased feeding would result in higher body burdens, as shown for *Diporeia* spp., an aquatic amphipod [40]. However, the feeding rate of the oligochaete *S. heringianus* was not sensitive to organism density [41], but much higher animal densities (30,000–134,000 worms $m^{-2}$) were used, compared to densities in this study or those normally found in nature [1,42,43]. Further, it seems unlikely that the relative beaker size or amount of sediment had significant impact on the observed variation with organism density. The exposures were carried out in the same size beakers for the 1:5 and the 1:50 ratios of animal dry weight:SOC (400 ml) but at differing amounts of sediment, whereas both

beaker size and amount of sediment were the same for the 1:10 and the 1:100 ratios of animal dry weight:SOC. Thus, a standard organism density relative to the SOC content should be established to ensure that exposures are similar among bioassays. Without such standardization, the exposure and the bioaccumulation will vary for the same sediment and contaminant concentration. The optimal density is uncertain, because the mechanism of the differential accumulation remains unknown.

The larger elimination constant with the nonlinear fit of the accumulation data at the trace concentration (Table 4) may have resulted from a change in pyrene bioavailability. If bioavailability declines, the flux into the organism will decline, even if the measured sediment concentration remains constant. This depletion of the bioavailable fraction of pyrene may occur, even if there is no change in the whole sediment concentration. Changes in bioavailability over time also help explain the decreasing body burden in accumulation experiments after 48 h for the trace-concentration exposures. Similar decreases have been observed for pyrene accumulation in the amphipod *Diporeia* spp. [29,44]. If a rate constant ($\lambda$) for the decline in bioavailability is included, Equation 1 can be changed to [44]

$$C_a = \frac{k_s \cdot C_s^{(t-0)}}{(k_e - \lambda)} (e^{-\lambda t} - e^{-k_e t}). \quad (3)$$

Fitting equation 3 to the accumulation data and using the measured elimination rate coefficient $k_e = 0.0256\ h^{-1}$, the $k_s$ becomes 0.067 ($\pm$0.003) g sediment $g^{-1}$ organism $h^{-1}$ and 0.058 ($\pm$0.003) g sediment $g^{-1}$ organism $h^{-1}$ with the 1:10 and 1:50 ratios of animal dry weight:SOC, respectively. In both animal densities, $\lambda$ has a value of $0.002 \pm 0.0003\ h^{-1}$, which is the same as reported earlier for pyrene in *Diporeia* spp. studies that used sediment collected from the same station in Lake Michigan [29,44]. The loss in bioavailability may be due to changes in partitioning of pyrene over time, the small decline in measured pyrene concentrations, and the small decline in radiopurity from 99.3% to approximately 92%.

Another possible reason for the observed peak in body burden at 48 h in the trace-concentration exposure could be the depletion of one or more readily bioavailable fractions of pyrene, such as pore water. It could be that the main route for accumulation during the first 24 to 48 h is pore water. If the accumulation rate of pyrene from water to the animals is greater than the desorption rate of pyrene from particles to the water, the concentration of pyrene in the pore water will decline, which means that the accumulation rate from water will decline and the importance of this route will also decline. Thus after 48 h, the net flux of chemical is temporarily out from the animal (i.e., $k_e C_a$ is higher than the combined $k_s C_s$), and the body burden reaches a new, lower steady state.

The $\lambda$ value incorporates all the factors that contribute to the apparent loss in bioavailability. Animal lipid content is also a factor that affects the apparent bioaccumulation of the chemical. During these exposures lipid content of *L. variegatus* declined (Table 3). The lipid-normalized pyrene concentration in the animals produced an accumulation curve with no decrease in concentration over time (Fig. 4B), as was observed for the wet-weight-normalized concentrations after 48 h at trace-concentration exposure (Fig. 4A). The nonlinear fit of the lipid-normalized data to Equation 1 gives uptake clearances of 5.93 ($\pm$0.42) g sediment $g^{-1}$ lipid $h^{-1}$ and 6.59 ($\pm$0.46) g sediment $g^{-1}$ lipid $h^{-1}$ for the animal densities 1:10 and 1:50, respectively (Table 4). The elimination rate constants are 0.053 ($\pm$0.004) $h^{-1}$ and 0.041 ($\pm$0.003) $h^{-1}$ for the animal densities 1:10 and 1:50, respectively (Table 4). These $k_e$ values are closer, but still approximately a factor of two greater, than the measured $k_e$ values, which suggests that the change in animal lipid content contributed significantly to the apparent change in bioavailability. Based on the model, the sediment conditions described above are still responsible for approximately half of the apparent change in bioavailability. These findings show that both the conditions in the sediment and the physiological state of the animals should be monitored throughout exposure to properly evaluate bioaccumulation.

Further, examination of the lipid-normalized uptake curve (Fig. 4B) continues to suggest a biphasic accumulation. As previously suggested, the contribution from pore water may be a more significant source early in the experiment; then, after initial depletion, the desorption-limited uptake from pore water becomes of similar magnitude to ingestion uptake. The flux from these two routes may well be lower than the initial pore-water uptake, resulting in a true reduction in bioavailability.

The high pyrene concentrations in the toxicity assay, which had more comparable $k_e$ values from nonlinear regression estimation than the measured elimination, showed less potential for bioavailability changes. The lipid normalization did not change elimination rates as dramatically (Table 4). Moreover, an initial pore-water contribution to the toxicokinetics seems less important than it is at the trace-treatment concentration. Both events may be related because the pore-water concentration for the toxicity assays would be predicted at or near saturation. Because it takes a long time for pore-water–particle systems to achieve equilibrium, the pore water may not have been at equilibrium as the concentration approached saturation. Thus, the relative importance of rapid pore-water accumulation relative to the total may have been minimized. Alternatively, the change in bioavailability represented by $\lambda$ may affect only a small amount of compound and be masked by isotopic dilution at high pyrene concentrations.

Elimination of pyrene by *L. variegatus* was much more rapid in the sediment than in the water-only elimination study. Higher elimination rates for feeding vs. nonfeeding organisms have been observed for several invertebrates [45–47] and were extreme for the amphipod *Diporeia* spp., which exhibited no measurable elimination of anthracene in the absence of feeding [48], but elimination was detected after only 8 h in sediment. Thus, enhanced elimination in the sediment was not unexpected but has major implications for performing gut purging in bioaccumulation studies.

Purging animals of ingested sediment before analysis is common, because ingested sediment in the gut of animals will contribute to the analyzed body burden. However, when animals are allowed to empty the gut in clean sediment before body-burden analysis, the estimate of tissue concentrations will likely be too low, because during the purging organisms may depurate a considerable amount from their true body burden and add weight in the form of uncontaminated sediment. In this case, *L. variegatus* excreted half of its body burden within 27.1 h. Further, simple purging in water-only may not completely remove the sediment or, if carried out too long or with ingestion of uncontaminated material, may enhance elimination. Either of the above experimental designs leads to undefined errors in body-burden measurements. Therefore, we suggest the following approach to assess the importance of gut sediment content. The natural logarithms of body burden in elimination data are plotted against time to obtain a straight line. Excluding the zero point, data are regressed for all other time points and extrapolated to the true body burden at time zero (the $y$ intercept of the regression lines; Fig. 6). Include only data from times the gut is expected to have been emptied. To calculate the body burden at time zero of elimination, back-transform the $y$ intercept and take into account the bias generated by these transformations and the use of natural log-transformed variables [49]. Body burden at time zero can be calculated with the following equation:

$$BB(0) = e^{y} \cdot e^{(MSE/2)}, \quad (4)$$

where

BB(0) = calculated body burden at time zero
$y$ = $y$ intercept from the regression
MSE = mean square of the error from the regression [49].

If the calculated body burden to elimination at time zero is lower than the measured body burden, the difference can be assumed due to extra compound associated with the sediment in the gut and/or loosely sorbed to the outside of the organism. In this study, sediment elimination data suggest that 20% of pyrene body burden in *L. variegatus* was due to gut content. In the water-only elimination data, the amount is 11%. The difference between these two estimates is likely attributed to the additional weight of uncontaminated material in the gut of the worms feeding on clean sediment. The additional weight without additional contamination would suppress the estimated body burden. Thus, the 11% contribution for pyrene is thought to be the more appropriate value. This value is the same or lower than values reported for heavy metals in some benthic organisms [24,25]. On the other hand, Oliver [26] reported that gut sediment content did not contribute any significant amount to body burdens of oligochaete worms for some chlorinated hydrocarbons. Hare et al. [25] suggested that 48 h would not be long enough for mayflies reared in clean water to clear their guts. Purging animals in clean water for a few hours may not realistically estimate the true body burden of benthic organisms. However, the latest results for *L. variegatus* using polydimethylsiloxane (PDMS) as a nonaccumulable tracer suggest that 10 h might be long enough for them to empty their guts even in water-only exposure (unpublished data). Thus, to estimate the importance of ingested sediment to body burden, a full elimination experiment must be performed and, if necessary, the extrapolation method used to correct the data—too time-consuming and expensive to conduct with bioaccumulation studies for regulatory use. Thus, the relation between gut contribution for a species and compound characteristics needs to be established so that appropriate corrections can be made without purging. This approach should reduce any opportunity for bias imposed by the length or conditions of the purging process.

Overall, *L. variegatus* accumulated sediment-associated pyrene rapidly and achieved apparent steady state within 48 to 168 h. Further, the bioaccumulation potential depended on organism density, but the mechanism is unknown. Thus, for developing standardized bioaccumulation studies, the density of organisms needs to be standardized, perhaps at a ratio of 1:50 animal dry weight:SOC. At high-pyrene treatment concentrations, the worms exhibited significant sediment avoidance, which reduced accumulation and likely minimized the observed mortality response. This is confirmed by the internal concentration, which reached only 2.3 $\mu$mol $g^{-1}$ as a peak with 17% mortality. Even after 1.5 months' storage, the bioavailability was still apparently changing, based on the estimate of $k_e$ from nonlinear regression compared to direct measures. Some toxicokinetic behavior can be explained by the decreasing lipid content in the animals, particularly at the trace-treatment concentration. However, the initial pulse of accumulation from the pore water remains a significant contributor to the apparent biphasic accumulation, even after lipid normalization. Finally, a full elimination study with extrapolation to the initial body burden is the only way to ensure that biases are not created in the results due to incomplete elimination of gut contents, and to account for the body burden losses of the purging process.

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